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# *Liquid transport fuels from microbial yeasts – current and future perspectives*

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Christopher J. Chuck,<sup>a\*</sup> Fabio Santomauro,<sup>b,c</sup> Lisa A. Sargeant,<sup>a</sup> Fraeya Whiffin,<sup>a</sup> Tanakorn Chantasuban,<sup>b</sup> Nur Rlnah Abdul Ghafter,<sup>b</sup> Jonathan L. Wagner<sup>a</sup> and Roderick J. Scott<sup>c</sup>

<sup>a</sup> Centre for Sustainable Chemical Technologies, Dept. of Chemical Engineering, University of Bath, Bath, BA2 7AY

<sup>b</sup> Dept. of Chemical Engineering, University of Bath, Bath, BA2 7AY

<sup>c</sup> Dept. of Biology and Biochemistry, University of Bath, Bath, BA2 7AY

\*email C.Chuck@bath.ac.uk, tel: +44(0)1225 383537, fax: +44 (0)1225 386231

## **Abstract**

Global transportation is one of the major contributors to GHG emissions. It is essential therefore, that renewable, carbon neutral fuels are developed to reduce the impact of this sector on the environment. Yeasts, especially *Saccharomyces cerevisiae*, are key to transforming renewable bioresources to fuels that can be used with little adaption to the current transport infrastructure. Yeasts demonstrate a large diversity that produces a great metabolic plasticity, as such, yeasts are able to produce a range of fuel-like molecules including alcohols, lipids and hydrocarbons. In this article the current and potential fuels produced through fermentation, the latest advances in metabolic engineering and the production of lipids suitable for biodiesel production are all reviewed.

## Key technical terms

Key term	Definition
Metabolic engineering	A method of optimising the regulatory processes within cells, used to produce high amounts of desirable compounds
Oleaginous yeast	Oil containing yeast, typically the triglyceride oil should be above 20% of the dry weight
Advanced biofuels	Fuels which are compatible with current fossil fuels, and tend to give higher performance than either bioethanol or biodiesel
Pentoses / hexoses	C <sub>5</sub> and C <sub>6</sub> sugars respectively
Isoprenoids	Diverse range of compounds, derived from isoprene (2-methyl-1,3-butadiene) units

## Future perspective

Within 10 years a number of key advances could potentially make advanced fuels derived from yeast a central component of the global energy mix. It seems probable that legislation will be enacted to increase the biofuel content in current transportation fuels, while at the same time ensuring more evidence-based green credentials for biofuels on the market. Strict fuel properties legislation is slowly being relaxed to allow alternatives to ethanol and biodiesel to enter the fuel market, this is likely to continue and within a decade it is probable that a larger range of fuels will be certified for general use. From an engineering perspective, advances in the processing, enzyme production and the development of novel strains of yeast will continue to reduce the total costs of converting lignocellulose to fuel molecules. Finally, a range of genetic toolkits are being developed for non-*Saccharomyces* yeasts, expanding the range of fuels and increasing the sugar to product conversion ratio. It seems likely therefore that alternative, more robust yeast strains to *Saccharomyces* will become prevalent for industrial biofuel production.

## Executive Summary

**Introduction:** Yeasts are capable of converting highly functionalised carbohydrate feedstocks into a range of potential fuel molecules such as alcohols, lipids and hydrocarbons. Over 1300 species of yeasts have been identified though it is *Saccharomyces cerevisiae* which remains one of the most widely used organisms for biotechnological applications.

**Feedstocks for yeast culture:** Central to the economic production of fuels from yeasts is a renewable source of feedstock to culture the yeasts. Lignocellulosic feedstocks are difficult to breakdown and offer a number of challenges that have limited the scale up of this technology. Current industrial processes generally use a pretreatment stage, followed by enzymatic hydrolysis, fermentation and separation. Developments over the last decade have reduced the costs of the processing substantially, and a number of demonstration plants are in operation that can produce lignocellulosic ethanol.

**Advanced biofuels through metabolic engineering:** While bioethanol is currently the most prevalent biofuel produced globally a range of alternative fuels with superior fuel properties are being developed using a number of yeasts. These include, longer chain alcohols and unsaturated hydrocarbon precursors that can be chemically upgraded to suitable fuel molecules. In general, bacteria have faster growth rates than yeasts, a higher metabolic plasticity and a wider range of available genetic tools. However, yeasts, in particular *S. cerevisiae*, have a higher tolerance to solvents and conditions often encountered on an industrial scale, have wider optimal pH ranges and a natural resistance to bacteriophages. These key factors have promoted a wealth of research into using *S. cerevisiae*, in addition to other suitable yeasts, to produce advanced biofuels.

**Oleaginous yeasts:** An alternative to fermentation fuels are lipid derived fuels such as biodiesel or hydrogenated fatty acids. While algal lipids have been heavily researched, a range of oleaginous yeasts are also capable of producing lipids. Over 20 species of oleaginous yeast have been identified and unlike algae, the lipid profile is simple and predominantly made up of palmitic acid, oleic acid and linoleic acid. The amount of lipid and its profile is highly dependent on the species and the growth conditions, though most yeast lipid will also contain other soluble components, such as sterols that can cause issues in the processing to suitable biofuels.

## Introduction

Due to growing pressure to reduce greenhouse gases and concerns over the increasing scarcity of fossil fuels, replacing liquid transport fuels with more sustainable alternatives is a key challenge of the 21<sup>st</sup> century [1-3]. Yeasts are capable of converting chemically functionalised and oxygenated biological compounds into a range of potential fuel molecules and could play a significant role in the production of sustainable biofuels. Yeasts are a large family of single-celled eukaryotic microorganisms of the kingdom fungi, comprising over 1300 identified species [4, 5], representing perhaps as little as 1% of the total number of extant species. Yeasts have long been of interest to geneticists: in 1997, *Saccharomyces cerevisiae* was the first eukaryotic organism to have its genome fully sequenced [6]; and *S. cerevisiae* remains one of the most widely used organisms for biotechnological applications [7, 8]. Whilst genomic information is available for only a small number of yeasts, there is clearly a great diversity in their physiology despite having only around 6000 genes. This is reflected in the diversity of biological niches inhabited by yeasts, which encompass the surface of fruit to the oceans [6]. This diversity is associated with great metabolic plasticity, which enable yeasts to produce a range of compounds suitable as fuels, including alcohols, triacylglycerides and more recently alternative biomolecules with the potential as drop-in fuels for the road and aviation sectors [9, 10].

Central to the economic production of fuels from yeasts is a viable source of sugar feedstock. The conversion of sugars and starches is well established globally. In 2011, this equated to over 10% of the world's supply being diverted for bioethanol production [11]. However, only a fraction of the land needed to produce these feedstocks is available for fuel production and to meet demand then second generation cellulosic technologies must be developed. Typical second generation feedstocks include grasses, forestry waste, agricultural stover and food waste. It has been estimated that, globally, 5.2 billion tonnes of biomass can be available for less than \$60 per tonne by 2030 [12], much of this derived from agricultural waste from the 2.3 billion tonnes of grain produced worldwide in 2011 [13]. The economic processing of the lignocellulosic feedstock is essential, and the latest advances in this area have been reviewed thoroughly elsewhere,[14] including a focus on the current challenges [15, 16], the necessary pretreatment stages [17] and the inhibitors formed from the cellulosic refining process [18].

## Yeast Derived Fermentation Fuels

### Production of bioethanol

Bioethanol is the most prevalent biofuel produced globally, with the vast majority being produced from the fermentation of sugars derived from terrestrial crops such as sugarcane and corn [19]. In 2011 over 68m tonnes of bioethanol were produced worldwide, with 87% being produced by the USA and Brazil [201]. All spark ignition vehicles produced in the US, after 1988, have been able to run on E10 (10 vol% ethanol in gasoline), in some cases this is rated up to E20. In Brazil all gasoline is sold with between 18 and 25 vol% bioethanol as of 2011. Bioethanol is mainly produced from corn in the USA and sugar cane in Brazil. Sugar cane is highly productive and generally produces  $6640 \text{ L ha}^{-1}$ , more than sugar beet which generally produces around  $5100 \text{ L ha}^{-1}$  and far in excess of corn, which produces  $3770 \text{ L ha}^{-1}$  of bioethanol [20].

There is limited data on the efficiency of production from lignocellulose, mainly due to the complexity of the process and the heterogeneous nature of the feedstock. A simplified flow diagram for a corn stover to ethanol plant is given in figure 1.

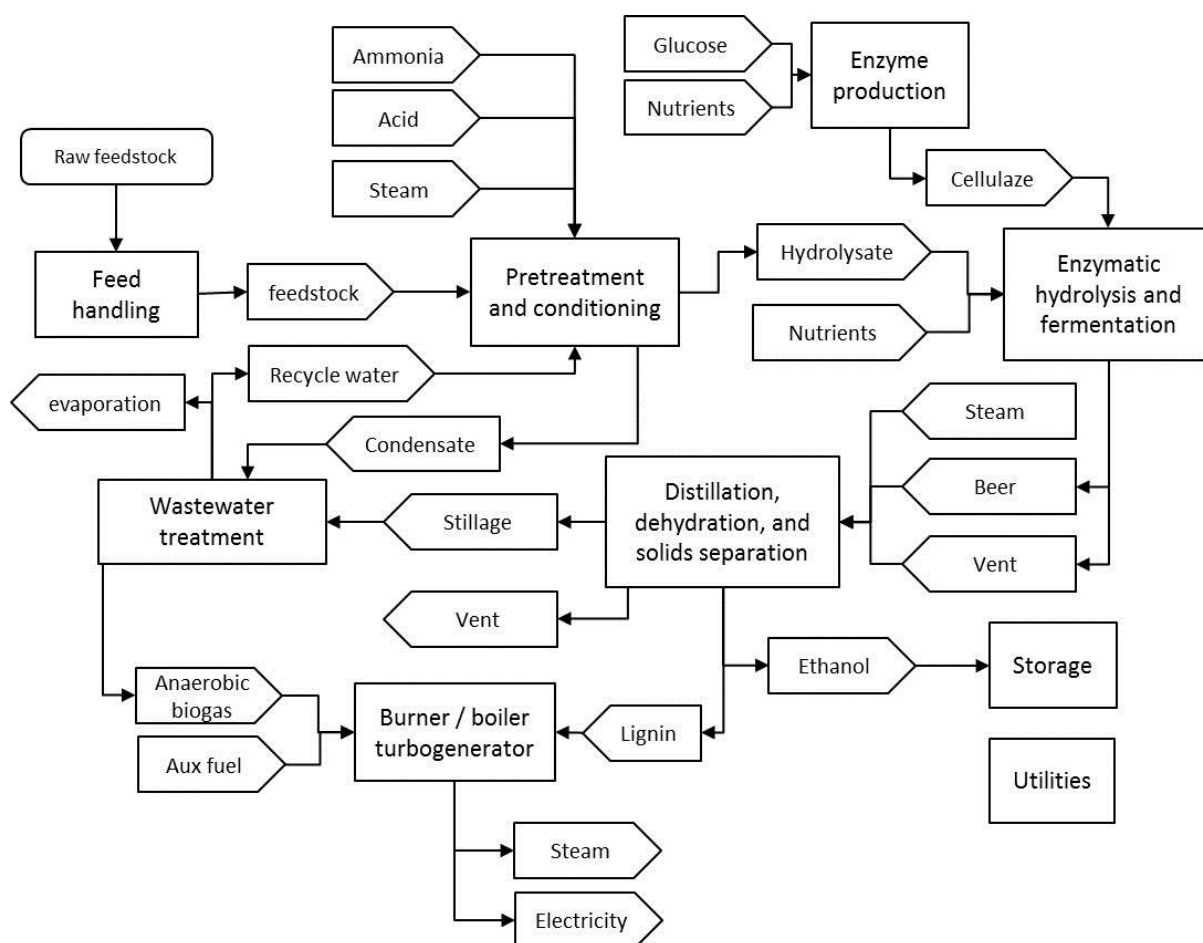


Figure 1 Simplified overall NREL flow diagram for a corn stover to bioethanol process plant, adapted from reference [21]

Many microorganisms such as yeasts, fungi and bacteria can produce ethanol from metabolising sugar feedstocks through fermentation. Under anaerobiosis, the respiratory chain is unable to work and the excess of reducing equivalents produced during glycolysis is recycled through the reduction of acetaldehyde to ethanol. The vast majority of bioethanol production processes use *Saccharomyces cerevisiae* because of its resilience to industrial conditions. Wild type *S. cerevisiae* can metabolise glucose and a range of disaccharides such sucrose and maltose and, while a range of yeasts display a reasonable tolerance to ethanol, such as *Hanseniaspora* spp., *Metschnikowia* spp. and *Pichia* spp., this is rarely more than 5 wt% [22]. In contrast, *S. cerevisiae* can tolerate up to 23 wt% ethanol [23]. Primarily it is this ethanol tolerance that has made *S. cerevisiae* central to the bioethanol industry. This characteristic is a complex trait that seems to be affected by the oleic acid content of the cell, the activity of enzymes like mitochondrial super oxide dismutase, the accumulation of protective metabolites like trehalose and the influence of metabolic pathways like

tryptophan synthesis [24-26]. So while *S. cerevisiae* cannot metabolise pentoses the yeast is also well understood, robust and highly tolerant to industrial conditions [27].

One of the key processes developed for cellulosic bioethanol is the Simultaneous Saccharification and Fermentation (SSF). In SSF, cellulose hydrolysis and fermentation are carried out simultaneously in the same reactor to produce bioethanol. The process uses smaller reactor sizes and reduces production of inhibitors [28]. The microorganisms commonly used in SSF are *Trichoderma reesei* and *S. cerevisiae*. Saccharification is usually the rate-limiting step, since the temperature for hydrolysis is non-optimal further to avoid killing the fermenting microbes. Further issues relate to the ethanol intolerance of the microorganisms and inhibition of enzymes by ethanol [29]. Since *S. cerevisiae* cannot assimilate the pentoses released by the hydrolysis of hemicellulose, alternative processes use yeasts that do have this capacity such as *Pichia stipitis* and *Candida shehatae*. While these yeasts do assimilate more sugars, their ethanol productivity and tolerance is far inferior to *S. cerevisiae* [30]. Consequently, these yeasts are generally used in conjunction with *S. cerevisiae*, in a process termed Simultaneous Saccharification and Co-fermentation (SSCF) [31]. While these processes have been developed mainly for use with *S. cerevisiae* to produce bioethanol, they are equally applicable to alternative yeast cultures to produce lipids from oleaginous yeasts or alternative fermentation products.

### Genetic engineering of yeasts for advanced biofuel production

The synthesis of advanced biofuels by microorganisms requires their modification by metabolic engineering to produce the desired components in industrially relevant quantities. Both yeasts and bacteria have desirable characteristics, such as an ability to grow on inexpensive substrates, and relatively easy manipulation due to an advanced knowledge of their genetic machinery. In general, bacteria have faster growth rates than yeasts, a higher metabolic plasticity and a wider range of available genetic tools. However, yeasts, in particular *S. cerevisiae*, have a higher tolerance to solvents and conditions often encountered on an industrial scale, have wider optimal pH ranges and a natural resistance to bacteriophages. Consequently, *S. cerevisiae* is generally preferred by industry for the production of recombinant proteins and metabolites [27, 32-34].

For yeasts, the genetic engineering of a metabolic pathway at its transcriptional, translational and/or post-translational level, represents the preferred strategy to achieve the exploitation and optimisation of a strain appropriate for industrial purposes [35]. A range of potential biofuels have been produced by the metabolic engineering of yeasts, predominantly *S. cerevisiae*, these include alcohols, short chain fatty acid esters and isoprenyl-derived biofuels (table 1).



**Table 1 Potential biofuels produced through the metabolic and genetic engineering of yeasts**

Fuel or fuel precursor	Yeast derived precursor	Melting point (°C)	Viscosity (mm <sup>2</sup> s <sup>-1</sup> )	Flash point (°C)	Yeast	Reference
Short chain fatty acids	-				<i>Saccharomyces cerevisiae</i>	[36]
1-butanol	-	-90	3.64 (20 °C)	35	<i>Saccharomyces cerevisiae</i>	[37]
2- butanol	-	-101.9	4.94 (20 °C)	28	<i>Saccharomyces cerevisiae</i>	[38]
2-methyl-1-butanol	-	-115	4.53 (20 °C)	24	<i>Saccharomyces cerevisiae</i>	[39]
3-methyl-1-butanol	-	-117.2 °C	4.59 (25 °C)	43	<i>Saccharomyces cerevisiae</i>	[39]
Farnesol	-	< 25	21.17 (20 °C)	96	<i>Candida albicans</i>	[40]
2-propanol	-	-89	2.49 (25 °C)	13	<i>Candida utilis</i>	[41]
Bisabolane	Bisabolene	<-78	2.91	108 111	<i>Saccharomyces cerevisiae</i>	[42]
Farnesane	Farnesene	<-47	2.33 (40 °C)	110	<i>Saccharomyces cerevisiae</i>	[43]
Pinene dimer	Pinene	-62 - -55	18-30 x 10 <sup>3</sup> cP (based on viscosities of variety of hydrogenated, mixed terpene dimers) (-15°C)	33	<i>Saccharomyces cerevisiae</i>	[27]
1-isopropyl-4-methyl cyclohexane	Limonene	-74	1.15 (25 °C)	50		
Further Sesquiterpenoids					<i>Saccharomyces cerevisiae</i>	[38]
Isoprene		-143.95 °C	0.29 (32 °C)	-54	<i>Saccharomyces cerevisiae</i>	[44]

Like ethanol, butanol and its branched isomers are gasoline substitutes and can be blended into gasoline at levels as high as 85%. Due to their longer chain length, these molecules have a higher energy density than ethanol, lower water susceptibility and are less corrosive. The production of n-butanol has been attempted in *S. cerevisiae* through the expression of a synthetic metabolic pathway using galactose as the carbon source and leading to n-butanol through the intermediates acetoacetyl-CoA, 3-hydroxybutyryl-CoA and crotonyl-CoA [37]. Over the course of this study, combinations of the isoforms of pathway enzymes were derived from different organisms including *E. coli*, *S. cerevisiae*, *Clostridium beijerinckii* and *Ralstonia eutropha*. The overexpression of the native isoform of thiolase, Erg10, and a NADH dependent 3-hydroxybutyryl-CoA dehydrogenase, Hbd, from *C. beijerinckii* was the most promising of those trialled.

In an alternative study the production of n-butanol from glycine was achieved using engineered *S. cerevisiae* [45]. The key enzyme of the pathway was a glycine oxidase (goxB) derived from *B. subtilis* which converts glycine to glyoxylate. Through the action of the endogenous enzymes, malate synthase and  $\beta$ -isopropylmalate dehydrogenase,  $\alpha$ -ketovalerate is produced. This molecule can then be converted to either n-butanol or irreversibly isomerised to  $\alpha$ -isoketovalerate by an isomerase, yielding iso-butanol. Iso-butanol has also been produced by a modification of the valine synthesis pathway in *S. cerevisiae* [46]. In this study, the authors overexpressed a 2-ketoisovalerate decarboxylase from *Lactobacillus lactis*, KivD, and a native alcohol dehydrogenase, Adh6, two enzymes leading to the production of iso-butanol. Carbon flux was also modified by deleting the gene for a major pyruvate decarboxylase, Pdc1, and overexpressing Ilv2, an acetolactate synthase and the first enzyme of the pathway. As a result of these two latter modifications, part of the carbon flux used in ethanol production was channelled towards the valine pathway because the pyruvate accumulating into the cell is converted by Ilv2 into 2-acetolactate.

The production of isopropanol has also been reported in *Candida utilis* through the use of pathways from *Clostridium spp*, more specifically *C. acetobutylicum* and *C. beijerinckii* [41]. The synthesis of isopropanol starts in this case from acetyl-CoA being converted to acetoacetyl-CoA by the native acetyl-CoA acetyltransferase ERG10. Then the acetoacetyl-CoA transferase from the genes *cfta* and *cftb* of *C. acetobutylicum* convert it into acetoacetate which is then decarboxylated to acetone and reduced to isopropanol. Also, the authors noted that the overexpression of ERG10 and the isoform ACS2 of acetyl-CoA synthase (not affected by glucose-induced degradation like isoform ACS1) improves significantly the isopropanol production.

The production of branched alcohol is uneconomically low, requiring novel strategies to increase productivity. One reportedly successful method compartmentalised the metabolic pathway into the

mitochondria [39] resulting in a 3-fold increase in iso-butanol production. The authors of this study concluded that the improvement was due to both the greater local enzyme concentration and the greater availability of pathway intermediates. Confining all the enzymes to an organelle apparently also reduced the cost of molecular transport across membranes and reduced competition for intermediates from other metabolic pathways. Iso-pentanol and 2-methyl-1-butanol were also produced in higher concentrations due to ILV genes expressing enzymes involved in valine, leucine and isoleucine synthesis, making the key intermediates  $\alpha$ -ketoisocaproate and  $\alpha$ -keto-3-methylvalerate available [39].

A similar strategy was followed by Atzumi *et al.* to synthesise the branched-chain higher alcohols, 1-propanol, iso-butanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol in *E. coli* [47].

The authors suggest that their method of increasing the amount of 2-keto acids converted to the related alcohols by a ketoacid decarboxylase and alcohol reductase, is also applicable to yeasts. In particular, they refer to KivD (ketoacid decarboxylase) from *L. lactis* and Adh2 (alcohol reductase) from *S. cerevisiae*, which both exhibit broad substrate specificity. For isobutanol and 3-methyl-1-butanol production, their study involved overexpression and manipulation of the valine and leucine biosynthesis respectively. For 1-propanol, 1-butanol and 2-methyl-1-butanol the overproduction of the intermediate 2-ketobutyrate was highlighted as the key factor. This was achieved by the insertion of the *IlvA* gene, coding for a Threonine deaminase. The 2-ketobutyrate can then be converted by KDC and ADH directly into 1-propanol, 1-butanol via 2-ketovalerate through the norvaline biosynthesis pathway or into 2-methyl-1-butanol via 2-keto-3-methyl-valerate in the isoleucine pathway.

Short chain fatty acid esters, fatty alcohols and alkanes are an important class of biofuels, which have a common origin in the synthesis of fatty acids (FAs) [48]. Consequently, increased FA biosynthesis is a sensible target for metabolic engineering. Fortunately, FA biosynthesis is among the best known metabolic pathways. In yeasts, synthesis is accomplished by a multienzymatic system, the fatty-acid synthase (FAS), localised to the mitochondrion. FAS elongates a molecule of malonyl CoA through repeated cycles of condensation,  $\beta$ -keto reduction, dehydration and enol reduction. The liberation of the fatty acids from FAS is catalyzed by a thioesterase, before the molecule is converted to a triacylglycerol by a series of reactions, the last of which is catalysed by an Acyl-CoA:diacylglycerol acyl-transferase (DGAT). This enzyme has been established as important rate limiting step in FA accumulation since its overproduction in *S. cerevisiae* led to a 3–9-fold increase in

TAG production [49]. Courchesne *et al.* proposed that this was due to diacylglycerols being subtracted to phospholipid production for the synthesis of TAG by DGAT [50].

Other enzymes are important for increasing FA production in yeasts. For example, there is a strong correlation between increased activity of the ATP: citrate lyase (ACL) and a malate enzymes and the oleaginous properties of a yeast [51]. The effect of the malate enzyme on lipid production is due to the reduction of NADP<sup>+</sup> to NADPH on the conversion of malate to pyruvate. The resulting NADPH is used by the enzymes responsible for FA synthesis, such as acetyl-CoA carboxylase (ACC), FAS and ACL [52]. Blocking competing pathways, in particular  $\beta$ -oxydation, has also proved promising in the accumulation of TAG in *Candida tropicalis* [53]. On culturing *C. tropicalis* using alkanes, the impairment of the carnitine acetyl-transferase increased the concentration of di-carboxylic acids in the mitochondria by reducing the flux of these molecules to peroxisomes for  $\beta$ -oxydation [54]. However, blocking  $\beta$ -oxydation completely resulted in reduced cell growth [53].

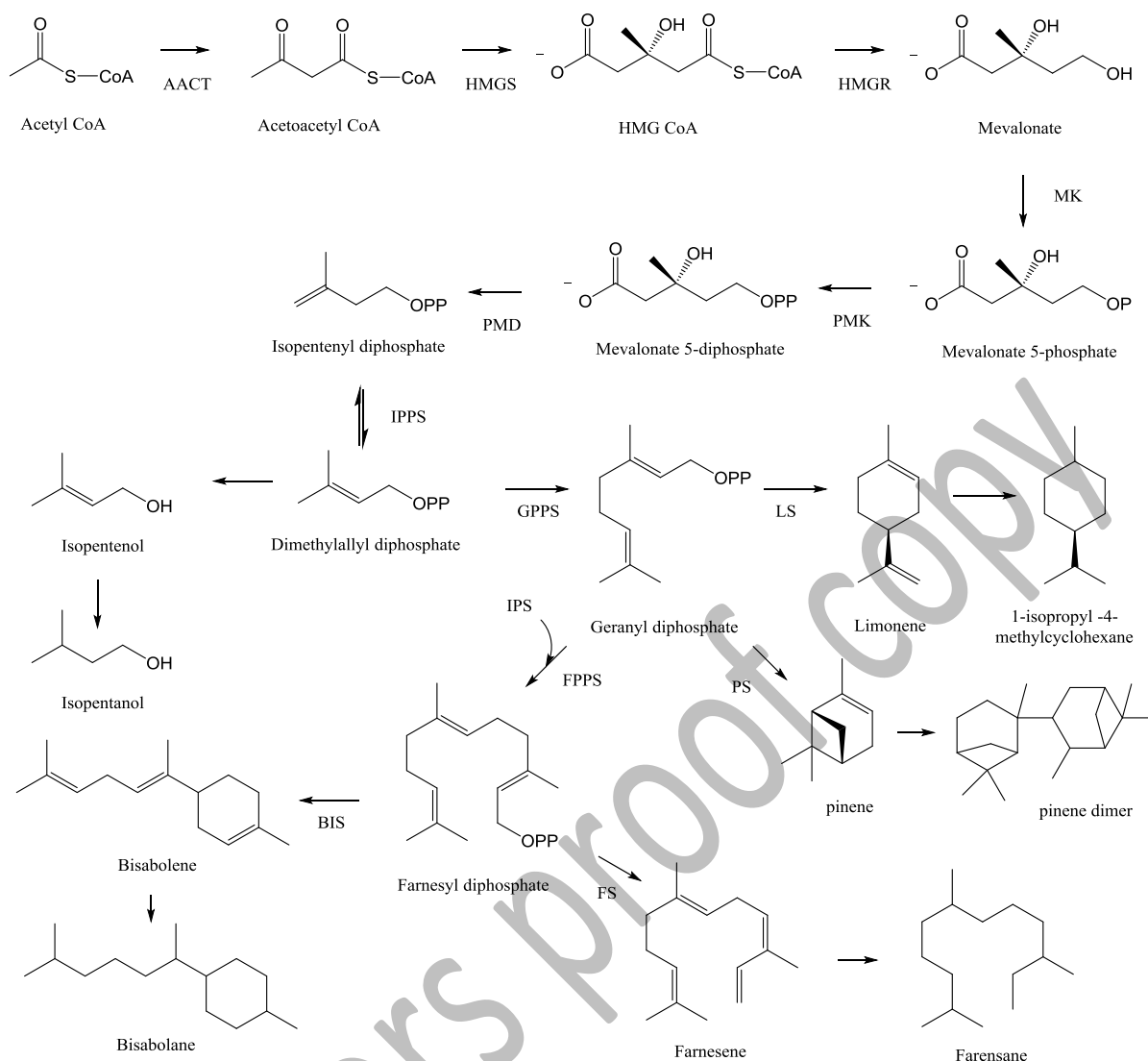
Other potential pathways to increased FA production are phospholipid synthesis and the production of oxaloacetate from phosphoenolpyruvate (PEP). Overexpression of DGAT (Acyl-CoA: diacylglycerol acyl-transferase) increases TAG production by diverting intermediates to phospholipid synthesis; this correlates well with the report that blocking phospholipid synthesis in *E.coli* results in the production of abnormally long FAs [55]. Decreased PEP activity in *Brassica napus* reportedly increases TAG concentration [56], and seems to play an important role in the regulation of lipid accumulation in microalgae [57].

The final class of biofuels produced by yeast is derived from the isoprenoids biosynthesis. Although the number of different compounds identified as isoprenoids (also called terpenoids) is extremely large, isoprenoids are generally recognized as molecules derived from the monomers isoprenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) and synthesised via the mevalonate pathway in yeasts and the deoxyxylulose pathway in bacteria [58]. These molecules are combined to make geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), the precursors of monoterpenes (C<sub>10</sub>), sesquiterpenes (C<sub>15</sub>) diterpenes (C<sub>20</sub>) and ergosterol. The metabolic engineering of the mevalonate pathway has aimed to increase the concentration of IPP and FPP, which, for example, can be directly converted to isopentenol and farnesol or into other molecules by several terpene synthases [59].

The isoprenoid synthesis in yeast is mediated by the mevalonate pathway, which has been extensively studied in eukaryotes. The main end product of this pathway is ergosterol, involved in the regulation of membrane fluidity and permeability. However, the intermediate metabolites serve

in the synthesis of other essential products like quinones, dolichols and hemes, as well as isoprenylated proteins. For this reason, the mevalonate pathway is usually considered to be divided into two parts: the first where farnesyl diphosphate is synthesised from AcCoA and whose intermediates are shared with other pathways, and a later part leading to the synthesis of ergosterol.

The first part is the main target for metabolic engineering (figure 2). In *S. cerevisiae*, the first step of the pathway involves the condensation of 2 AcCoA molecules to give acetoacetyl-CoA, a reaction catalysed by the acetoacetyl-CoA thiolase (ERG10), which is regulated by the intracellular levels of sterols and other intermediates. The addition of a third AcCoA molecule to create 3-hydroxy-3 methyl glutaryl CoA (HMG-CoA) is accomplished by a HMG-CoA synthase (ERG13), also strictly regulated. The pathway continues with the synthesis of mevalonate from HMG-CoA through a HMG-CoA reductase, an enzyme present in two isoforms: HMGP1, which is very stable and expressed in aerobic conditions, and HMGP2, which has a rapid turnover and whose expression can then be easily tuned to the cellular conditions in anaerobiosis. The next step entails the double phosphorylation at the C<sub>5</sub> position of mevalonate, performed by a mevalonate kinase (ERG12) and phosphomevalonate kinase (ERG8), followed by a decarboxylation by a mevalonate diphosphate decarboxylase (ERG19) which in the end produces isopentenyl diphosphate. The final steps of the first part of the pathway involve the isomerisation of IPP to dimethylallyl pyrophosphate (DMAPP), the condensation of these two molecules to form geranyl diphosphate (GPP) which is then extended to form farnesyl diphosphate (FPP) by the condensation of a second molecule of IPP. These steps are performed respectively by an IPP isomerase (IDI1) and a farnesyl (geranyl) diphosphate synthase (ERG20).



**Figure 2 Metabolic pathways in yeast, used in the production of farnesene and other isoprenoid biofuels, adapted from Peralta-Yahya et. al. [27]**

A successful strategy to increase isoprenoid synthesis is reduced squalene synthase activity, this enzyme catalyses the cyclisation of FPP to squalene, a precursor of ergosterol [60]. However, since ergosterol is essential to the cell, the gene was put under the control of the promoter CRT3, which is inducible by copper, to enable some expression of squalene synthase. The overexpression and control of enzymes in the MVA pathways has also proved useful, in particular use of a truncated version of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (tHMG) lacking an N-terminal regulatory domain proved more kinetically active. FPP can also be converted to farnesene using an  $\alpha$ -farnesene synthase from the peel of apple (*Malus domestica*) fruits [61]. Additional enzymes, such as bisabolene synthase (AgBIS) from the plant *Abies grandis* [62], and amorphadiene synthase can convert farnesene to bisabolene and amorphadiene, respectively. The biosynthesis of pinene, a potential jet fuel molecule, first required the condensation of IPP and DMAPP in a head-to-tail

configuration to have GPP which was then cyclised to pinene by a cineole synthase from Greek sage (*Salvia fruticola*), which co-produces limonene [63]. However, due to the intrinsic toxicity of these molecules towards yeast, research is needed to optimise their production by this host.

Whilst increasing the production of specific advanced fuel molecules is an important target for genetic engineering, increasing the range of feedstock sugars to include xylose, arabinose, ramnose (from hemicellulose) and galacturonic acid (from pectin) is also highly desirable.

### Metabolic engineering of yeast for fermenting cellulosic sugars

The majority of studies in this area relate to the genetic engineering of yeast strains to improve xylose assimilation. In bacteria, xylose is usually converted to xylulose by a xylose isomerase, before being phosphorylated to xylulose-5-P that can enter main metabolism through the Pentose Phosphate Pathway (PPP). In yeasts such as *Scheffersomyces stipites* (formerly *Pichia stipites*), *Candida shehatae* and *Pachysolen tannophilus*, these first steps differ by involving the action of a NADPH-dependent xylose reductase (XR) followed by a NAD<sup>+</sup> - dependent xylitol dehydrogenase (XDH) [64]. However, the use of these yeasts on an industrial scale is prevented by their sensitivity to alcohol, furfural inhibitors, as well as their acute sensitive to low pH and low oxygen concentrations.

For this reason, *S. cerevisiae* has been preferred as a host for the expression of heterologous genes enabling the metabolism of xylose. For example, the xylose isomerase from *Thermus thermophilus* (xyl1) was successfully introduced into *S. cerevisiae* [65], though this represents one of the few successfully exploited bacterial genes. The authors suggest that this is due to the enzyme having a eukaryote-like substrate-binding domain. However, the yeast strain modified with xyl1 exhibited relatively poor fermentation performance, even when other enzymes from the same pathway were overexpressed. Since the enzyme was derived from a thermophile, this was presumably due to suboptimal temperatures in the fermentation [66].

A more successful strategy involved introduction of genes for XR (XYL 1) and XDH(XYL 2) from other yeasts [64, 67, 68]. *Scheffersomyces stipites* (formerly *Pichia stipites*) and *S. cerevisiae* strains possessing these genes were able to metabolise xylose. The authors reasoned that this was due to the special characteristics of XR, which can use both NADPH and NADH and therefore formed a recycle loop for the NADH produced by XDH. Previous work had already shown that the oxide-reduction balance is a key factor in metabolic engineering for fermentations [69]. However, a drawback of using the *S. stipitis* XR gene was the accumulation of NADH inside the cell leading to the production of xylitol and glycerol in anaerobiosis. This was due to the  $K_m$  of XR for NADPH being 10 times lower than for NADH, though this effect was overcome by expressing XDH and XR in a ratio of

15:1. An alternative study demonstrated that the XR gene from *Candida tenuis* engineered through directed evolution had a higher specificity for NADH than for NADPH [70].

While glucose and xylose are the most prevalent sugars from depolymerised lignocellulose, a number of other sugars, such as arabinose, are produced in small amounts. A number of metabolic pathways involved in the catabolism of arabinose have been reported for *Penicillium chrysogenum* and *Aspergillus niger* [71, 72]. However, metabolism by microorganisms is reasonably rare due to the redox imbalance caused by 2 NAD<sup>+</sup> - dependent and 2 NADPH- dependent reactions. The transformation of *S. cerevisiae* with genes for increased arabinose metabolism from both yeast and bacterial sources has been attempted [73, 74]. Arguably the most promising results were obtained following the insertion and overexpression of the codon-optimised ARA (arabinose) genes from *Lactobacillus plantarum*, and the endogenous genes of the pentose phosphate pathway, together with several cycles of directed evolutionary engineering through mutagenesis [75].

The sugars galacturonic acid and rhamnose are also produced from lignocellulose. *S. cerevisiae* is reportedly unable to grow on galacturonic acid, and only a handful of yeast species, but including *Candida* and *Pichia* species reportedly have this capacity [76, 77]. On the other hand, bacteria show a widespread ability to assimilate both of these substrates through a pathway that involves the conversion of D-galacturonate to pyruvate and glyceraldehyde-3-P, via the intermediate 2-keto-3-deoxy-6-phosphogluconate which consumes NADH and ATP.

However, the further conversion of pyruvate to ethanol in anaerobic conditions would require an additional NADH molecule, which again would affect the normal redox balance. To prevent this, galacturonic acid could be restricted to a minor fraction (up to 10%) in the feedstock. At this concentration, the excess in NADH found in anaerobiosis could be recycled rather than leading to the overproduction of glycerol.

Different pathways for the metabolism of rhamnose exist in bacteria and yeasts. In bacteria, rhamnose is usually converted to DHAP and L-lactaldehyde through a phosphorylated intermediate. In contrast yeasts convert rhamnose to pyruvate and L-lactaldehyde through redox reactions that increase the concentration of NADH. Under anaerobic conditions, *S. cerevisiae* converts L-lactaldehyde to 1,2-propandiol using NADH as a cofactor. This led Van Maris *et. al.* to suggest that the optimal strategy for rhamnose metabolism is the introduction of a yeast transporter from *Aureobasidium pullulans*, *Pichia stipitis*, or *Debaryomyces polymorphus* along with the bacterial pathway for rhamnose metabolism which doesn't create an excess of NADH. This approach was



attractive since the cell redox balance was not affected by an increase in NADH. As a consequence, the cell is not forced to produce glycerol to recycle this cofactor [78].

As discussed earlier, depolymerised cellulose also contains inhibitory compounds generated from the decomposition of the sugars in the hydrolysis stage. Success in selecting furfural and HMF-resistant *S. cerevisiae* strains has been achieved by serial culturing in media with increasing concentrations of the toxic compounds [79]. In this regard, the content in NADPH and NADH for the reduction respectively of furfural and HMF to furfuryl and hydroxymethyl furfuryl play a key role, as well as enzymes in the PPP [79, 80]. Phenolics are also a powerful inhibitor in lignocellulose hydrolysates and their negative effects have been counteracted by the transfection of a laccase from *Trametes versicolor* [81], or the overexpression of a phenylacrylic-acid decarboxylase [82]. While these compounds are well known to be highly toxic to *S. cerevisiae*, oleaginous yeasts tend to show a far greater tolerance [83, 84].

## Lipid Derived Fuels from Oleaginous Yeasts

Triglycerides, the main components of plant and animal lipids, are becoming increasingly popular as a feedstock for a range of industrial applications. The two dominant uses of glyceride lipids are in the food industry and for biodiesel production.

Biodiesel is a fatty acid alkyl ester produced by transesterification of glyceride lipids, and can be used as a replacement for petroleum-derived diesel fuel [85, 86]. Roughly 95% of biodiesel is produced from edible plant oils such as rapeseed, sunflower and palm oil [87]. However, concerns over the sustainability and competition with food have driven the search for additional glyceride feedstocks. One option is to produce fats and oils from microbes, commonly referred to as single cell oils (SCOs). Oleaginous microorganisms, including microalgae, yeast, bacteria and moulds can accumulate lipids to more than 20% of their dry weight [88]. The microbial lipids are mainly composed of triglycerides [51], but may contain free fatty acids, other neutral lipids such as mono- and diacylglycerides and sterol-esters, sterols and polar lipids e.g. phospholipids, sphingolipids, glycolipids [89, 90].

Microbial oils offer many advantages over vegetable oils including a short life cycle, they are less labour intensive, and less affected by location, season and climate change [91, 92]. While production of algal oil, and its conversion into useable fuels has been demonstrated [93-95], the commercialisation of this technology remains elusive. Unsustainable demands on freshwater and fertilisers, scarcity of low-cost concentrated CO<sub>2</sub> and high energy requirements for algae culture collectively hinder the commercialisation for biofuel production [96, 97]. Algae also require light of appropriate intensity and wavelength. Consequentially, production is affected by the amount of light

incident at potential production sites, which limits the supply of suitable culture locations. Although algae culture does not require prime agricultural land, the current low levels of productivity ( $4 \text{ kg m}^{-3}$ ) would result in a large land footprint. Whilst artificial lighting would improve productivity, this, as well as the need for temperature control (algae must be cooled during the day and heated at night) adds to the energy costs. The presence of a cell wall of variable toughness also makes extracting the oil energy intensive [98-101].

In contrast, yeast grow do not require light, have shorter doubling times and reach much higher cell densities ( $10\text{-}100 \text{ g l}^{-1}$  in 3-7 days) [92]. They also produce a wide range of fermentation products in addition to oils, which is economically attractive to potential biorefinery plant. Of particular interest to care product applications is the synthesis by yeast of biologically derived surfactants (biosurfactants) e.g. sophorolipids, which are biodegradable [102, 103-106]. Many of the charged lipids have interesting properties, such as antimicrobials and anti-proliferatives from polyol lipids in *Rhodotorula glutinis*.

SCOs from heterotrophic organisms have attracted attention since the 1980s during a period where there was a shortage in cocoa butter and a SCO with a similar lipid profile was sought [107]. The yeast *Yarrowia lipolytica* reportedly produces a suitable lipid substitute [108]. Of over 1600 known yeast species, 40 are known to be oleaginous. These yeasts belong to ascomycete genera *Candida*, *Cyberlindnera*, *Geotrichum*, *Kodamaea*, *Lipomyces*, *Magnusiomyces*, *Metschnikowia*, *Trigonopsis*, *Wickerhamomyces*, and *Yarrowia* [109-111] and to basidiomycete genera *Cryptococcus*, *Guehomyces*, *Leucosporidiella*, *Pseudozyma*, *Rhodosporidium*, *Rhodotorula* and *Trichosporon* [109, 112-116].

While the percentage of lipid accumulation within the SCO is of importance, the lipid coefficient, i.e. gram of lipid produced per gram of sugar consumed is one of the most important parameters for microbial biofuel production. The sugar stream resulting from hydrolysed lignocellulosic substrates are a mixture of hexose and pentose sugars, predominantly these are glucose and xylose that are present in a ratio of 2:1 [117]. Given that a large proportion of the costs of producing oil from oleaginous microorganisms comes from the cost of the initial feedstock, it is vital that both sugars are utilised for the economics to be favourable. If all of the sugar consumed was directed towards lipid synthesis, a maximum lipid yield of  $0.32 \text{ g/g}$  and  $0.34 \text{ g/g}$  can be produced from glucose and xylose, respectively [118]. However, due to other cellular processes requiring glucose, even under ideal conditions for lipid production, the lipid yield on glucose is very rarely more than  $0.22 \text{ g/g}$  [51] [119]. For example, when *T. cutaneum* was cultivated on equal quantities of glucose and xylose, the lipid coefficient was found to be  $0.17 \text{ g/g}$ , a slight decrease from  $0.20 \text{ g/g}$  when glucose was the sole

carbon source [117]. However, when grown in a fed-batch system, the lipid coefficient for *R. toruloides* reached as high as 0.24 g/g [120]. As the sugar is also used for the production of oil-free biomass, there is a fine balance between the percentage of oil accumulated within the cell and the overall biomass yield. Based on this, an oil content of a minimum of 40% dry weight has been proposed to be necessary [121].

While there has been little economic analysis undertaken on the production of biofuels from SCOs, it is understood that the production costs derive largely from the initial feedstock costs, as well as extraction and conversion costs, but the largest contributor comes from the fermentation process itself. It seems unlikely that with present yields and technology, a yeast SCO could be produced for less than £1500/tonne [119].

### Lipid extraction

Following the growth and accumulation of the lipids, the microorganisms first have to be harvested or separated from the culture medium. This involves removing large quantities of water and thus the harvesting of biomass can contribute to 20-30% of the total biomass production costs [121]. Common harvesting methods include sedimentation, centrifugation and ultra-filtration. Flocculation can be used to aggregate cells. Whilst extraction techniques for yeast and algae are very similar, harsher conditions are required for algae due to the relatively resilience of the cell. Therefore, whilst the techniques described below are applicable to both yeast and algae, adaptations are required.

Organic solvents are commonly used to extract lipid from microbial biomass, including hexane, methanol, ethanol, chloroform and diethyl ether [122]. The rate of extraction is influenced by factors such as particle size, type of solvent, temperature and agitation [87]. One of the most widely used methods is that developed by Bligh and Dyer, which uses chloroform and methanol [123], though industrially n-hexane is generally favoured [87].

First used for extraction in the 1980s, microwave-assisted extraction is a fast and efficient extraction method used for solid-liquid extraction. Rapid generation of heat and pressure within the cell forces the lipid out of the biological matrix, enabling a high recovery of the target molecules [124]. Whilst this technique still relies on the use of solvents, the volume is often markedly less than traditional solvent extraction, and the pressure produced within the microwave chamber can be varied depending on the volume and the boiling point of the solvent [122, 125]. Cryogenic grinding, supercritical fluids, pressurised liquids, acid/base treatment, enzyme lysis or other mechanical disruption have also been reported as methods to extract lipids from the cell [126]. Sonication has also been widely used as a method for disrupting microbial cells which uses a cavitation effect to

crack the cell wall/membrane. Furthermore, bead beating has been used on a laboratory and industrial scale to cause direct damage to cells using fine beads and high-speed mechanical spinning.

### Lipid properties

Aside from the triglycerides, other lipid soluble compounds are synthesised by yeast including terpenes, hydrocarbons, sterols and phospholipids. While often being minor constituents, they can have significant effects on the fuel properties. For example, sterol glucosides which have a high melting point ( $>240\text{ }^{\circ}\text{C}$ ) and limited solubility in biodiesel have been identified as causing precipitate formation when stored at low temperatures [127, 128]. These precipitates can lead to fuel filter plugging. This emphasises the importance of aligning the oleaginous yeast with the intended application, as in the case of *M. pulcherrima*, the sterol content can be as high as 3.5 %d.wt [129].

Like algal oils the lipid might require further chemical refining to give a pure triglyceride feed. The triglycerides can then be converted into biodiesel, in a similar method to vegetable oils. Biodiesel has many advantages over its mineral diesel equivalent, including renewability, low sulphur content, no aromatic content, biodegradability, reduction of many exhaust emissions, high flash point, and inherent lubricity [130]. However, poor low temperature properties, low oxidative stability and a slight increase in the production of nitrous oxides (NO<sub>x</sub>) reduce the blend level in diesel [131]. In the USA, biodiesel is often used as a B20 blend (20% biodiesel, 80% diesel), whereas in the European Union this value is decreased to B5-7 to comply with governmental regulations [131].

The chain length, degree of unsaturation and branching of the lipid all affect the physical properties of the oil. The biodiesel produced must therefore comply with the existing regulatory standards, predominantly ASTM 6751-02 and EN 14-214, in the US and EU respectively. These often serve as guidelines for the development of standards elsewhere. The physical properties of biodiesel are heavily dependent on the fatty acid profile. The resulting composition largely influences the cetane number, kinematic viscosity, oxidative stability, and cold flow properties of the fuel [48, 132, 133]. Generally saturated esters have poor low temperature properties, high viscosity but excellent cetane numbers, whereas polyunsaturates have low melting points and low viscosity but also have severely reduced cetane numbers (table 2) [85].

*Table 2: Properties of fatty acid methyl esters found in yeast biodiesel, data adapted from references [85] and [134]*

Property	Fatty acid methyl ester				
	16:0	18:0	18:1	18:2	18:3
Cetane number	81[183]	89[183]	62[183]	42[183]	22.7
Kinematic viscosity (40°C; mm <sup>2</sup> /s)	4.38	5.85	4.51	3.65	3.14
Oxidative stability (110°C; h)	>24	>24	2.79	0.94	0.00
Density (g/cm <sup>3</sup> ) 15°C	0.867[183]	0.868[183]	0.87746	0.89016	0.90166
40°C	0.8491	-	0.85937	0.87195	0.883
Melting point (°C)	28.5	37.7	-20.2	-43.1	-45.5

Plant-based oils used for the production of biodiesel (e.g. soybean, rapeseed, canola) contain primarily C<sub>16</sub> and C<sub>18</sub> fatty acids, with varying degrees of unsaturation [135]. This is also similar for biodiesel derived from yeasts, though yeast oils tend to be lower in polyunsaturates such as linolenic acid found in plant oils. In contrast, the greater amount of oleic acid ester (18:1) generally found in yeast oils will be advantageous for oxidative stability without compromising on the cold flow properties [133].

*Table 3: Relationship between structure and biodiesel performance parameters, adapted from reference [116]*

	Cetane number	Melting point	Oxidative stability	Kinematic viscosity	Heat of combustion (greater is better)
<b>Chain length</b>	Longer gives higher	Shorter are lower	NR	Shorter is less viscous	Longer gives greater
<b>Degree of unsaturation</b>	Saturated gives higher	Saturated gives higher	Saturated is more stable	Unsaturated is less viscous	Unsaturated is lower
<b>Branching</b>	Branching gives slightly lower	Branched FFAE has lower	NR	Branched is less viscous	Branching gives slightly lower

While there are many reports focussing on the fuel properties of biodiesel derived from microalgae, there are relatively few studies that examine the properties of biodiesel produced from oleaginous yeasts [136]. In general, algal biodiesel has a much more complex fatty acid profile in comparison to plant and yeast oils, with alkyl chains ranging from C<sub>12</sub> – C<sub>22</sub> [121]. Similarly to yeast, the lipid composition ranges depending on species and growth conditions for the algal culture [135]. Algal oils are highly polyunsaturated, with eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6) commonly found. While this will beneficially increase the cloud point of the fuel, it has detrimental

effects on the oxidative stability of the fuel. As such, lipids with four or more double bonds are limited to a maximum of 1% mol., and more specifically, the linolenic acid (18:3) content of the fuel is limited to 12 mol% for vehicle use within the European Union.

The total unsaturation of the fuel is indicated by its iodine value, with the EN 14214 biodiesel standard cannot exceed an iodine value of 120 g iodine / 100 g biodiesel [93]. Due to this, it is likely that the fuel will need to be hydrogenated to improve its properties as well as to decrease the probability of the fuel polymerising in the engine oil. Furthermore, algal oil contains the lipid-soluble Mg-containing chlorophyll complex. While only present in small quantities, if used at scale the presence of magnesium in the fuel could have detrimental effects on the engine performance and longevity.

Recently, *Wahlen et al.* compared biodiesel derived from bacteria, algae and the yeast *Cryptococcus curvatus* [137]. The fuels were then tested for their emissions and performance on a 2-cylinder research engine. The lipid profile of the yeast biodiesel used composed of 60% oleic acid (18:1), 15% palmitic acid (16:0), 18% steric acid (18:0) and 5% linoleic acid (18:2). The kinematic viscosity of this fuel measured  $4.5 \text{ mm}^2 \text{ s}^{-1}$ , within the range for both ASTM D6751 and EN14214 specifications. Both the energy density and heating value of the fuel were similar to the commercial soybean biodiesel, whereas the biodiesel cetane index was 67, considerably higher than its biodiesel equivalents. This is comfortably above the 51 minimum for EN14214 specification.

**Table 4: Current selected specifications in biodiesel standards (ASTM D6751 in the United States; EN 14214 in Europe) caused by the fatty acid composition and heteroelements**

Specification	ASTM D6751	EN 14214
Cetane Number	47 min.	51 min.
Kinematic viscosity	$1.9\text{-}6.0 \text{ mm}^2 \text{ s}^{-1}$	$3.5\text{-}5.0 \text{ mm}^2 \text{ s}^{-1}$
Oxidative stability <sup>a</sup>	3 h min	6 h min
Cloud point	Report	-
Cold filter plugging point	-	<sup>b</sup>
Cold soak filtration test	360 s max	-
Sulfur	0.0015 mass% (15ppm) max for ULSD	10.0 mg kg <sup>-1</sup> max
	0.05 mass% for 500 ppm sulphur diesel	
Na + K combined	5 ppm ( $\mu\text{g g}^{-1}$ ) max	5.0 mg kg <sup>-1</sup> max
Ca + Mg	5 ppm ( $\mu\text{g g}^{-1}$ ) max	5.0 mg kg <sup>-1</sup> max
<sup>a</sup> Rancimat test per standard En 14214. <sup>b</sup> Depends on geographic location and time of year.		

Table 5 Examples and culture conditions for oleaginous yeasts. *T* Temperature, *t* time, MnM mineral medium, MM minimal medium, F fermentor, f flask, F-b fed-batch, CF continuous fermentor, SL sophorolipids, SF solid fermentation, MEL mannosylerythritol lipid, OP optimum. Table adapted from Ageitos et. al. [102] and Santamauro et. al. [129]

Species	Dry biomass (g/L)	Lipid content (%)	<i>T</i> (°C)	<i>t</i> (h)	pH	Culture
<i>Apiotrichum curvatum</i> UfaM3	15	45.6	30	–	5.5	CF
<i>A. curvatum</i> Ufa25	15	40	30	150	5	F
<i>A. curvatum</i> ATCC 20509	85	35	30	70	4.8	F
<i>A. curvatum</i> ATCC 20509	15.11	47	32	145	5.5	F
<i>Candida</i> 107	18.1	37.1	30	3,528	5.5	CF
<i>C. bombicola</i> ATCC 22214	–	SL 21 g/L	26	120	6	F
<i>C. bombicola</i>	29	SL 41 g/L	30	190	7	F
<i>C. curvata</i> D	10.6	27	28	72	5.5	CF
	8.2	30	28	72	5.5	CF
<i>Cryptococcus curvatus</i>	91	33.3	28	75	5.5	Surer®
<i>C. albidus</i> var. <i>aerius</i> IBPhM γ-229	–	63.4	OP	–	5	F
<i>C. albidus</i> var. <i>albidus</i> CBS 4517	26.78	46.3	20	90	5.5	CF
<i>C. curvatus</i> ATCC 20509	118	25	28	50	5.5	F F-b
<i>C. curvatus</i> ATCC 20509	18.4	49.7	30	24 + 72	5.4	F
	16.1	68.9	30	24 + 72	5.4	F
<i>C. terricolus</i>	16	39	25	184	5.5	F
<i>Lypomyces lipofer</i> IBPhM γ-693	–	51.5	OP	–	5	F
<i>L. starkeyi</i>	20.5	61.5	30	120	6	F
<i>L. starkeyi</i> DSM 70295	13.3	56.3	30	220	5	F
	9.3	72.3	30	220	5	F
<i>L. starkeyi</i> AS 2. 1390	18	30	28	96	5.8	F
	20.9	20.5	28	96	5.8	F
	14	24.9	28	96	5.8	F
<i>M. Pulcherrima</i>	7	40	15	360	5	f
<i>Pseudozyma aphidis</i>	33	MEL 75 g/L	27	288	6.5	F F-b
	30	MEL 110 g/L	27	228	6.5	F F-b
<i>Rhodospiridium toruloides</i>	18.2	76.1	30	120	6	F
<i>R. toruloides</i> Y4	151.5	48	30	600	5.6	f F-b
	106.5	67.5	30	134	5.6	F F-b

<i>R. toruloides</i> AS 2. 1389	6.9	42	28	96	5.8	F
	7.2	26.8	28	96	5.8	F
	4.8	16.8	28	96	5.8	F
<i>R. toruloides</i> ACT 10788	–	79	27	168	5	F
<i>R. toruloides</i> Y4	127.4	61.8	30	140	5.6	F F-b
<i>Rodotorula glutinis</i> IIP-30	22.3	66	30	120	4	F
<i>R. glutinis</i> IIP-30	17.2	39	30	120	4	F
	25.0	42	30	120	4	F F-b
<i>R. glutinis</i> NRRL y-1091	185	40	–	–	5.5	F F-b
<i>R. glutinis</i> AS 2. 703	5	30.2	28	96	5.8	F
	6.9	12	28	96	5.8	F
	4.3	4.9	28	96	5.8	F
<i>R. gracilis</i> CFR-1	10	68	28	120	5	F
<i>R. gracilis</i> CFR-1	13.7	59.4	–	–	5	SF
	13.9	60.3	–	–	5	SF
<i>R. minuta</i> IIP-33	15	48	30	80	4.5	F F-b
<i>Trichosporon cutaneum</i> AS 2. 571	3.2	65.6	28	96	5.8	F
	4.2	13.4	28	96	5.8	F
	5.6	8.2	28	96	5.8	F
<i>Yarrowia lipolytica</i> LGAM S(7)1	8.7	40	28	240	6	F
<i>Y. lipolytica</i> ACA-DC 50109	15	44	28	120	6	F
<i>Zygosporomyces lactosus</i>	–	66.5	OP	–	5	F

### Fatty acid profile

Unlike algal oils, in general yeast lipid is composed of C<sub>16</sub> and C<sub>18</sub> fatty acids. Palmitic acid (C<sub>16</sub>:0) constitutes 15-25% w/w of the total lipid, while palmitoleic (Δ<sup>9</sup> 16:1) is found in concentrations generally less than 5% w/w. Oleic acid (Δ<sup>9</sup> 18:1) is the principal lipid accumulated in yeast cells, sometimes higher than 70% w/w, whereas stearic acid (18:0) and linoleic acid (Δ<sup>9,12</sup> 18:2) are minor components of the oil, found in concentrations of 5-8% w/w and 15-25% w/w, respectively [89]. Polyunsaturated lipids such as α-linolenic acid (18:3), are not commonly synthesised in yeast oils.

### Effect of growing conditions

The key step to lipid accumulation in oleaginous microorganisms is the change in the intracellular concentration of various metabolites, due to the exhaustion of some nutrients in the culture medium [89]. In many of the studies performed, lipid accumulation is initiated when nitrogen is depleted and it becomes the limiting factor of microbial growth, but a carbon source is abundantly available. Nitrogen exhaustion initiates a series of metabolic steps leading to de novo lipid



biosynthesis in which the carbon source is used for lipid accumulation rather than cell proliferation processes (for reviews see: Ratledge and Wynn [51]; Ratledge [138]). The most important of these pathways involves the cleaving of citric acid into acetyl-CoA and oxaloacetate by the enzyme complex ATP-citrate lyase (ATP-CL). ATP-CL is only present in oleaginous microorganism and it is therefore considered to be the most important factor accounting for the oleagenicity of these microorganisms [139]. Lipid accumulation is influenced by the C/N ratio, with lipid accumulation induced at molar ratio C/N > 20 [8], but with an optimum being close to 100 [102]. Indeed, the lipid yield from *R. glutinis* more than doubled when the C/N ratio was increased from 20 to 70, but a further increase to C/N 120 did not lead to higher lipid yields [140]. It should be noted however, that nitrogen limitation is not the sole factor for the accumulation of lipids during the growth of the oleaginous microorganisms. For example, lipid accumulation in *Rhodospiridium toruloides* was induced in nitrogen-rich medium when sulfate or phosphorus became the limiting factor of cell growth [141, 142].

Inhibitors from the decomposition of lignocellulose, are well known for their toxicity to microorganisms such as *S. cerevisiae* [7], though tend to be less inhibitory to oleaginous yeasts [83, 84]. The oleaginous yeast species that have been tested for inhibitor tolerance are *Rhodotorula glutinis*, *Trichosporon cutaneum*, *Rhodotorula rubra*, *Rhodospiridium toruloides*, *Lipomyces starkeyi*, *Cryptococcus albidum* and *Trichosporon fermentans*. Some of these species, most notably *Trichosporon fermentans* and *Trichosporon cutaneum* have shown elevated tolerance to HMF, furfural and acetic acid [83, 143].

### Tailoring the lipid profile

Wu et al. demonstrated that by changing the carbon-to-sulfur (C/S) ratio of the growth medium, the fatty acid composition of the resulting oil from *R. toruloides* could be tailored accordingly. For example, a higher C/S molar ratio favoured the production of saturated fatty acids [141].

The growth temperature also influences the degree of saturation of the lipids. Temperature-induced variations in the fatty acid profile of the yeast *C. oleophila*, *C. utilis* and *R. toruloides* have been demonstrated, but the effects were species specific [144]. Reducing the culture temperature for *C. curvatus* increased the amount of saturated esters by 10% [145], while the amount of polyunsaturates in various yeast of the Zygomycete genera were reduced substantially at lower growth temperatures [146]. In contrast, lower incubation temperatures were reported to increase the level of polyunsaturates in *R. glutinis* [136, 147] and *C. lipolytica* [148].

### Alternative lipid derived fuels

To improve the performance of lipid derived fuels a range of novel processes are being developed. These fuels have a far more similar composition to diesel than traditional FAME. For example, one process is hydrotreating, which involves the deoxygenation of triglycerides into linear chain alkanes, over a metal supported catalyst [149, 150]. This process must then be followed by additional isomerisation and cracking reactions over Pt or Pd catalysts on zeolite supports. This process improves the low temperature properties of the resulting alkanes (HFA or HEFA) fuels [151]. Generally the hydrogenated fuel has a high cetane value of between 84 and 99, which creates a superior diesel product. Depending on the degree of isomerisation the fuel also has a low cloud point (as low as -30 °C) and as there are no double bonds the fuel is extremely stable. While microbial oils could be used in this process there is some indication that molecules such as sterols and terpenes can deactivate the hydrogenation catalysts used in this process, and would need to be refined prior to use [301-303].

### Conclusions

Yeasts play a key industrial role in the conversion of renewable biomass to liquid fuels. While generally bacteria have faster growth rates and are simpler to genetically transform, most yeasts, in particular *S. cerevisiae*, have a higher tolerance to industrial conditions and solvents used on this scale, have wider optimal pH ranges and a natural resistance to bacteriophages. Due to these factors a range of alternative, high performance fuels are being developed through genetic and metabolic engineering of *Saccharomyces* and non-*Saccharomyces* yeasts. One promising feedstock for biofuel production is lipids that can be converted into alkane fuels of fatty acid methyl esters. Over 20 species of oleaginous yeast are known, and offer a credible alternative to terrestrially derived vegetable oils or algal lipid feedstocks.

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